Distinct functions for Ras GTPases in the control of proliferation and apoptosis in mouse and human mesangial cells

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In previous work, we have demonstrated that Ras GTPases regulate proliferation in a range of human renal cells. The present work compares human and mouse mesangial cell (HMC and MMC) responses to specific knockdown of Ras genes with antisense oligonucleotides (AS-oligos), and examines the role of the p21 (cip1) and p27 (kip1) cyclin-dependent kinase inhibitors in these responses in mouse cells. HMC and MMC were lipofectin transfected with ras-targeted AS-oligo at 200–400 nM for 18 h followed by growth of cells in 20% serum for 18–72 h. Cell proliferation was assessed with an MTS assay and bromodeoxyuridine (BrdU) uptake. Apoptosis was quantified using nuclear stain with Hoechst 33342 dye. In MMC, Ha-ras AS-oligo caused an increase in apoptosis from $<2\%$ to 10–15% of cells after 18 h in serum ($P<0.01$). Control, Ki-ras and N-ras AS-oligos had minimal effects on apoptosis. BrdU uptake studies showed that BrdU + ve MMC were increased by 20–40% ($P<0.05$) after Ha-ras AS-oligo at 24 h; other ras AS-oligos were inactive. HMC number was reduced by 40–80% ($P<0.01$) at 48–72 h by both Ha-ras and Ki-ras AS-oligos. These actions were associated with reductions in BrdU + ve cells. In HMC, the ras AS-oligos did not induce apoptosis. p21(−,−) MMC showed exaggerated apoptotic responses to Ha-Ras AS-oligo. In mouse cells, Ha-Ras expression appears necessary to prevent apoptotic cell death; Ras expression does not appear necessary for cells to progress through the cell cycle. In human cells, Ras does not appear necessary to prevent apoptosis but Ha-Ras and Ki-Ras appear to be required for cell cycle progression.

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Renal disease is often characterized by dysregulation of proliferation and apoptosis.[1] Abnormal mesangial cell proliferation is characteristic of several forms of human glomerulonephritis and is associated with excess glomerular cellularity, and with the damaging process of glomerular sclerosis.[2] An understanding of the cellular and molecular basis for this response and the cell signaling pathways involved is likely to lead to new targets for future therapies. There has been considerable progress in dissecting the likely extracellular factors that drive the proliferative response, and the mitogenic cytokine platelet-derived growth factor appears to have an important role.[3–5] There is less complete understanding of the intracellular signaling involved.

Ras monomeric GTPases play a critical role in the control of cellular proliferation, differentiation and apoptosis, and are potential targets in renal therapies.[6–9] There are three different isoforms of Ras: Harvey (Ha-Ras), Kirsten (Ki-Ras) and Neural (N-Ras). However, the distinct functions of these species are poorly understood. The upstream signals that control Ras activation include platelet-derived growth factor, fibroblast growth factor, epithelial growth factor and a range of other mitogens that have been implicated in renal disease. We have demonstrated in earlier work that both Ki-Ras and Ha-Ras are required for cell cycle progression in human renal fibroblasts.[7] The present work presents a study of the role of the Ras genes in mesangial cells.

In classical cell biological models, such as NIH 3T3 cells, there is abundant evidence for the role of the Ras family of monomeric GTPases in the control of cell proliferation.[6] The three ras genes are all potent transforming agents when mutated to be locked in the active GTP-bound form. Recent studies have shown that renal cell proliferation is also governed at the level of the cell cycle by regulatory proteins. Specifically, cyclin-dependent kinase (CDK) inhibitors, including p21 and p27, limit renal cell proliferation by binding to and inhibiting cyclin–CDK complexes.[10] However, the regulation of the CDK inhibitors p21 and p27 is not well understood in renal cells. There is recent evidence that the transcription of p21 and the expression level of functional p27 can be regulated by Ras in non-renal cells.[11–13] However,

in renal mesangial cells the relationships between the Ras isoforms and p21 and p27 have not been defined.

The goal of the current work was to determine the roles of the Ras GTPases in the control of proliferation and apoptosis in renal mesangial cells using ras antisense oligonucleotides (AS-oligos), and to examine the possible interactions between Ras and the CDK inhibitors p21 and p27. The results obtained show that the roles of Ras genes are quite distinct when mouse and human cells are compared. In human cells, the Ki-Ras and Ha-Ras GTPases are required for stimulated proliferation; in mouse cells, Ha-Ras is an antiapoptotic survival signal but Ras is not needed for proliferative signaling.

RESULTS

Cell number is altered by suppression of specific Ras isoforms

Figure 1 shows results determining the activity and specificity of the ras AS-oligos using linear-range reverse transcriptase-polymerase chain reaction (RT-PCR). Figure 1a displays RT-PCR products using Ki-Ras(4B) primers on mRNA from human mesangial cells (HMC) transfected with each of the four AS-oligos. Incubation with the Ki-Ras AS-oligo at 200 nM for 16 h abolished the Ki-Ras(4B) RT-PCR product. The 18S ribosomal bands were used to ensure equal loading in each sample. The Ha-Ras and N-Ras AS-oligos and control AS-oligo did not alter the levels of Ki-ras mRNA. Similarly, Figure 1b shows that the Ha-Ras AS-oligo specifically reduced Ha-Ras mRNA, which was not altered by the other AS-oligos. Figure 1c similarly shows that the N-Ras AS-oligo is specific in depletion of mRNA for its target isoform. These results demonstrate specific actions of the three ras antisense oligos on the levels of cellular mRNA for each isoform. Ras protein half-life has been determined by pulse chase to be 18–24 h, and the specific knockdown of Ras protein 48 h after transfection was found to be >80% (data not shown) as reported previously for renal fibroblasts.[7]

In mouse mesangial cells (MMC), the control AS-oligo was found to have no effect on the cell number curves when compared with lipofectin-alone mock transfection conditions. Figure 2a shows the cell number curves over 72 h for wild-type MMC after transfection with each of the three Ras AS-oligo compared with control AS-oligo transfection. As expected, there was an increase in the cell count with time under control conditions. This was not altered by transfection with N-ras or Ki-ras oligo. In contrast, there was a small but significant suppression of the growth curve at 24 h after Ha-Ras AS-oligo transfection but cell numbers were unaffected at 48 and 72 h.

The growth curves for p21(−,−) MMC are shown in Figure 2b. Cell number was not significantly altered in cells transfected with Ki-Ras and N-Ras AS-oligo compared with control AS-oligo. In contrast, there was significant growth suppression after transfection with the AS-oligo targeting Ha-Ras at 24, 48 and 72 h ($P<0.01$ versus control).

Figure 2c and d shows the results obtained in p27(−/−) and double knockout p21(−/−)p27(−/−) MMC, respectively. Transfecting these cells with each of the ras isoform AS-oligos did not significantly alter cell number at 24–72 h.

The cell number curves for HMC are shown in Figure 3. In human cells, the control AS-oligo caused a nonspecific reduction in cell numbers at 48–72 h. A nonspecific action

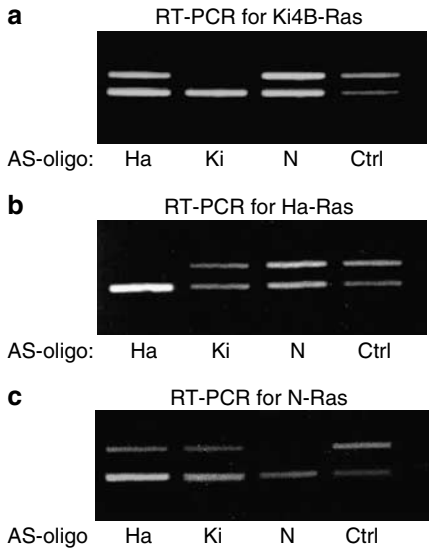

Figure 1 | Demonstration of specific actions of the ras oligos. In each PCR panel, the upper band represents ras and the lower band represent 18S ribosomal RNA control. (**a**) RT-PCR for Ki-ras after transfection with each of the three ras oligos (Ha-ras, Ki-ras, N-ras) and control oligo (ctrl). Similar results (**b**) for Ha-ras RT-PCR and (**c**) for N-ras are shown.

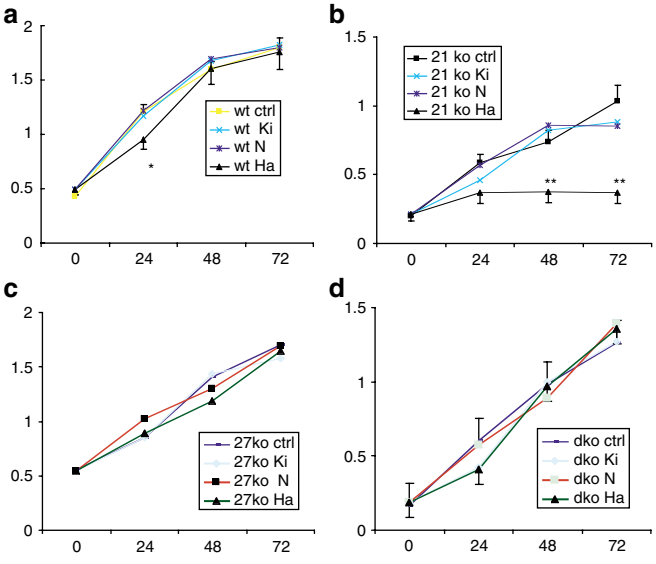

Figure 2 | Cell number as a function of time as monitored by the MTS assay for MMC, and the actions of Ras knockdown by isoform-specific oligos. The ordinates show cell number expressed as arbitrary units. (**a**) Data from wild-type MMC, (**b**) data from p21(−,−) MMC, (**c**) results for p27(−,−) MMC and (**d**) data for the double knockout p21(−,−) p27(−,−) MMC. Error bars are s.e.m. Each figure represents data from three independent experiments, each performed in quadruplicate ($n=12$). *$P<0.05$ versus control oligo data; **$P<0.01$ versus control oligo data.

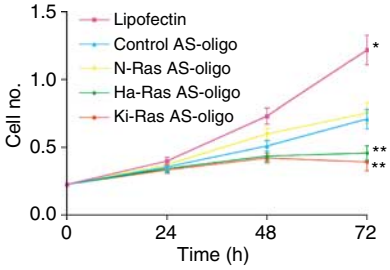

Figure 3 | Cell number as a function of time as monitored by the MTS assay for HMC, and the actions of Ras knockdown by isoform-specific oligos. The ordinate shows cell number expressed as arbitrary units. Error bars are s.e.m. Data are shown from three independent experiments, each performed in triplicate ($n = 9$). *$P < 0.05$ versus control oligo data, **$P < 0.01$ versus control oligo data.

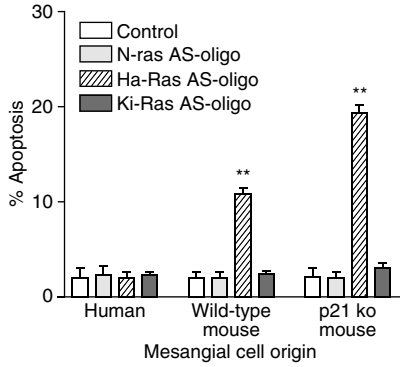

Figure 4 | Mean values for percentage apoptosis shown after transfection of the HMC and MMC by the ras oligos. Mock transfection with lipofectin (mock Tx) is compared to transfection with 200 nM Ha-ras, Ki-ras, N-ras and control oligos. In all, >150 cells were counted for each experiment, and the mean of three experiments is shown. Error bars are s.e.m. **$P <$ 0.01 versus control oligo for cells of the same genotype.

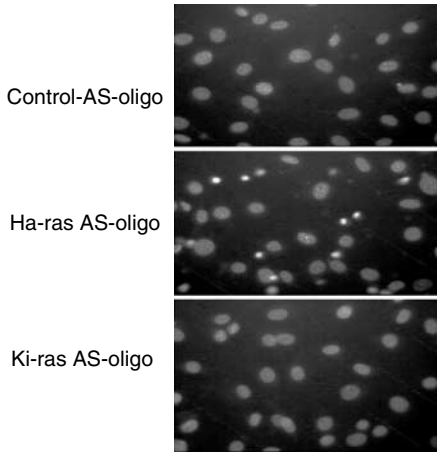

Figure 5 | Illustration of the actions of ras oligo on apoptosis in MMC. Cell nuclei are stained with Hoechst 33342 as described in the text.

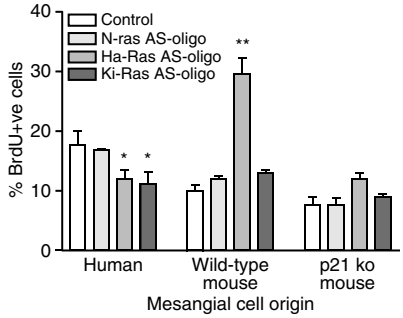

Figure 6 | Mean percentage of BrdU + ve cells after transfection with oligos targeting Ras isoforms Ha, Ki and N or with control oligo. Data for HMC are compared with wild-type and p21(−,−) MMC. In all, >150 cells were counted in each of three independent experiments. Error bars are s.e.m. *$P < 0.05$ versus control oligo.

has been reported previously with this control AS-oligo in human renal fibroblasts.[7] In contrast to wild-type MMC, transfecting HMC with the Ki-Ras and Ha-Ras AS-oligos further suppressed cell number at 48–72 h significantly. N-ras oligo did not alter cell number in HMC compared with the control AS-oligo.

Further work to define the effects observed was performed with the HMC and with the wild-type and p21(−,−) MMC.

Reducing Ha-Ras increases apoptosis in MMC

Figure 4 shows the levels of apoptosis in the wild-type and p21(−,−) MMC and in HMC 24 h after transfection with AS-oligos. In wild-type MMC, there was a clear increase in apoptosis (by more than fivefold) after transfection with the Ha-Ras oligo ($P < 0.01$ versus control AS-oligo-transfected cells), which was not evident in cells transfected with N-ras or Ki-ras oligo. In p21(−,−) MMC, there was a 10-fold increase in apoptosis after Ha-ras AS-oligo transfection. Ki-ras and N-ras AS-oligos did not induce apoptosis. In HMC, in contrast, none of the Ras AS-oligos had an effect on apoptosis. Figure 5 shows examples of wild-type MMC appearances under fluorescent microscopy, illustrating the apoptosis induced by Ha-Ras AS-oligo.

Ha-Ras regulates DNA synthesis

In order to determine whether the change in cell number was due to changes in DNA synthesis, bromodeoxyuridine (BrdU) staining was performed in AS-oligo-transfected and control cells. The effects of the Ras oligo on BrdU uptake are shown in Figure 6. In HMC, both the Ha-ras and Ki-ras oligo reduced the proportion of BrdU + ve cells by 20–40% ($P < 0.05$ versus control). In wild-type MMC, the Ha-ras AS-oligo caused a significant *increase* in % BrdU + ve cells; other Ras AS-oligos were inactive. In p21(−,−) MMC, the AS-oligos did not cause significant changes in BrdU uptake, although the Ha-ras AS-oligo led to a tendency toward higher values.

Ha-ras and the expression of pAkt and the CDK inhibitors p21 and p27

We next asked whether specific ras isoforms alter the expression of p21 or p27 and activation of the survival protein Akt in the wild-type MMC. Figure 7 shows Western

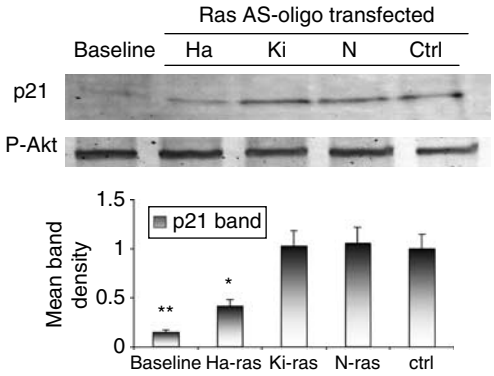

Figure 7 | Western blot of p21(cip1) and pAkt expression in wild-type MMC comparing the effects of ras oligos. Baseline data were obtained before serum stimulation. Other data were obtained after 24 h in 20% serum after prior transfection with oligos. Bar chart shows the mean densitometry of p21 bands normalized to the data in control oligo. Error bars are s.e.m. $n = 3$, $^{**}P < 0.01$ versus control oligo, $^{*}P < 0.05$ versus control oligo.

blot analysis demonstrating the effects of the Ras AS-oligos on the expression of p21 and phospho-Akt in wild-type MMC. As expected, the levels of p21 were increased from baseline in wild-type cells on serum stimulation under control conditions. This increase was partially suppressed by the Ha-Ras AS-oligo, but not altered by the Ki-Ras and N-Ras AS-oligos as quantified in the bar chart of Figure 7. The expression of p27 was variable and was not significantly altered by the different Ras AS-oligos (data not shown). The results of phospho-Akt blotting did not show any significant differences when the AS-oligos were compared.

DISCUSSION

These experiments on MMC showed a striking difference in the sensitivity of p21(−,−) cells to Ha-Ras AS-oligo when compared to the other genotypes. The normal growth curve is completely flattened after Ha-Ras AS-oligo transfection (Figure 2b). This action is explained by the dramatic increase in apoptosis caused by this Ha-ras AS-oligo (Figure 4) and does not appear to be related to any failure of cell cycle progression, as BrdU uptake was not suppressed by Ha-Ras oligo (Figure 6). The knockdown of Ha-Ras also caused clear but lesser increases in apoptosis in the wild-type MMC. This is reflected in the reduction in cell number at 24 h in the wild-type cells after Ha-Ras knockdown (Figure 2a). The cell number curve then returns to control levels at 48–72 h and this is likely to be related to a compensatory increase in proliferation in these cells, which is evident from the observed increase in BrdU uptake (Figure 6). These mouse wild-type mesangial cells appear to undergo an increase in proliferation, which compensates for the cells lost by apoptosis. These data specifically implicate Ha-Ras in the prevention of apoptosis in MMC in serum. The p21(−,−) cells are particularly sensitive to apoptosis following the loss of Ha-Ras and this is consistent with the observation that these cells are also more sensitive to the withdrawal of serum. P21(cip1) has been implicated in transforming growth factor-β responses and in the genesis of mesangial cell hypertrophy.[10,14] The present data suggest that p21 and Ha-ras act together in some way to ensure mouse mesangial cell survival in the presence of serum. Mouse cells null for p27 appeared to behave like wild-type cells in these experiments and p27 does not appear to influence the serum-induced proliferation of MMC either positively or negatively. In the light of this, it was surprising to find that the p21/p27 double knockout cells also behaved like wild-type cells in these experiments. It appears that the link between p21 and survival is uncoupled in the absence of p27. The reasons for this are not clear.

The actions of Ras oligo on p21 and p27 protein levels were small and may not have played a significant role in the effects observed. However, it is of interest that the well-described upregulation of p21 by serum was inhibited by Ha-Ras AS-oligo and not by the other Ras AS-oligos (Figure 7). Induction of p21 by certain cytokines (e.g. transforming growth factor-β) appears to be Ras-dependent but for others (e.g. platelet-derived growth factor) it is not.[15] The present data are consistent with the reported role of Ras GTPases in the control of p21 transcription[11,12,15] and specifically implicate Ha-Ras in this regulation. This result suggests that Ha-Ras alone is an important regulator of p21 protein levels.

Ras is classically thought to stimulate cell cycle progression and this action was observed in the HMC data where the knockdown of Ha-Ras and Ki-Ras reduced the percentage of cells in the S phase and reduced cell numbers in the MTS assay (Figures 3 and 6). These actions are exactly as reported in human renal fibroblasts.[7] However, no such action was observed in the mouse cells and indeed the percentage of cells in the S phase was increased by knockdown of Ha-Ras.

The different effects of Ras knockdown on the cell number curves in mice and humans (Figures 2 and 3) can be explained by differential actions on apoptosis and the cell cycle. In human cells, the knockdown of Ha-Ras or Ki-Ras is antiproliferative with no changes in apoptosis. Cell numbers are therefore reduced by Ha-Ras and Ki-Ras oligo. In mouse cells, the large increase in apoptosis on knockdown of Ha-Ras is partially offset by an increase in cell cycle progression, leaving the cell numbers relatively unaltered except at the early time point of 24 h. In mouse cells, Ki-Ras and N-Ras do not appear to modulate apoptosis or cell cycle progression.

A likely downstream effector in the protection from apoptosis exerted by Ha-Ras in MMC is the phosphatidylinositol 3′-kinase-phospho-Akt pathway.[16,17] We could not confirm a role for phospho-Akt, but cannot rule it out entirely as the extracted protein at 24 h may provide information only on the non-apoptotic cells and earlier time points might be needed to observe altered phospho-Akt preceding apoptosis. Another possible downstream modulator of the actions of Ha-Ras in mouse cells is the Ral-GDS system. Ha-Ras activity has been specifically linked to Ral-GDS, and activity in this pathway is known to be able to modulate apoptosis and cell differentiation.[18] This interaction requires further study in renal cells.

This work has highlighted the very clear differences in the functions of the Ras genes in HMC and MMC. There is existing evidence for biochemical and cellular differences in the functions of the indvidual Ras proteins Ha-, Ki- and N-Ras.[19,20] However, differences in Ras function when comparing mice and humans have not been reported before. In the context of renal therapies, the present results suggest that the study of mouse models of *in vivo* Ras antagonism may not provide a clear pathway to the development of therapies in humans. Moreover, the role of Ha-Ras as an antiapoptotic survival signal may need to be re-evaluated in human cells, as much of the present literature is based on murine and rodent cell work.

MATERIALS AND METHODS

Mesangial cell culture

HMC were obtained in primary culture from Clonetics Inc. Wokingham, UK and were maintained in RPMI 1640 medium with 10% fetal calf serum. All HMC experiments were carried out between passages 4 and 9. MMC were obtained in primary culture using published techniques utilizing wild-type mice of strain C57B6 and mice that were null for p21(cip), p27(kip1) and double (p21, p27 knockouts).[10] Genotypes were confirmed by PCR, and the lack of appropriate CDK-inhibitor expression in the knockout mesangial cells was confirmed by RT-PCR and Western blotting for p21 and p27. MMC were maintained in 20% fetal calf serum.

Oligonucleotide transfection

AS-oligos to individual human and mouse ras isoforms (Ki-ras, Ha-ras and N-ras) were phosphorothioate forms with 2'-methoxy-ethyl modifications supplied by Isis Pharmaceuticals (Carlsbad, CA). These chemical forms of oligo have been shown to be of low cell toxicity and to have half-lives in cell culture of over 72 h. A control AS-oligo was used that targets the HIV promoter region and has no sequence similarity to either mouse or human DNA; details of this oligo have been reported previously.[7] The specificity and activity of the AS-oligos were demonstrated using RT-PCR for the three ras mRNA transcripts as reported previously.[7] The oligos were transfected into the cells using Lipofectin™ (Gibco BRL, Paisley, UK) with 16–18 h of incubation at oligo concentrations of 200–400 nM. An initial mixture of lipofectin in 6–12 μl/ml OptiMEM (Gibco) was vortexed, and incubated at room temperature for 45 min. AS-oligos were then added and the vortexed mixture was left for a further 15 min before being added to the cells. The ratio of lipofectin to oligo was 0.3 μl/10 pmol in each case.

Cell proliferation and apoptosis

To study the effects of ras oligos on proliferation, HMC were rendered quiescent by serum starvation for 24 h, trypsinized and seeded in triplicate into 96-well plates (5000 cells/well). MMC were placed in 0.2% serum for 24 h. The rationale for this was that preliminary studies showed that the absence of serum caused significant cell loss particularly in the p21(−,−) mouse cells. Cells were transfected with oligos targeting the individual ras genes and with control oligos targeting part of the HIV-1 sequence as reported previously.[7] Viable cell numbers were determined by the MTS assay (Cell titer 96, Promega, Southampton, UK), measuring absorbance at 490 nm. The correlation between viable cell number and absorbance at 490 nm is linear.

To test the hypothesis that specific Ras isoforms regulate mesangial cell proliferation, DNA synthesis was assessed by BrdU uptake. Cells were grown in 35 mm dishes, serum-stimulated and transfected as described earlier. At 18 h after the addition of the serum, used as a source of growth factors, BrdU (10 μM) (Sigma, Gillingham, UK) was added to each dish for 16 h. Dishes were then washed with phosphate-buffered saline (PBS), and fixed for 45 min (3 volume 50 mM glycine and 7 volume ethanol). Cells were then incubated in 4 M hydrochloric acid for 10 min, washed three times in PBS and blocked in 5% goat serum/0.05% Tween/PBS for 15 min before being incubated overnight in anti-BrdU antibody (1:100 dilution) (Sigma) at 4°C. Cells were washed with PBS and incubated for 30 min with TRITC-labelled anti-mouse IgG antibody (Sigma). Cell nuclei were then counterstained with Hoechst 33342 at a concentration of 10 μg/ml for 15 min (Sigma). Cells were then visualized with a fluorescent microscope. BrdU + ve cells would stain nuclei red whereas all cell nuclei would stain blue. A total of 150 cells were counted from at least five different fields by an observer in a blinded fashion.

To study the role of Ras isoforms in mesangial cell apoptosis, HMC and MMC were grown in 35 mm dishes and transfected with oligos as described before for 18 h. Hoechst 33342 was added (10 μg/ml), and cells were incubated at 37°C for 15 min. Cell nuclei were then visualized under fluorescent microscopy. Apoptotic nuclei were then identified as showing characteristic signs of chromatin condensation and fragmentation. A total of 150 cells were counted from at least five different fields.

PCR and Western blotting

For the initial detection of Ras isoform mRNAs, 40 cycles of PCR were used. For human ras, the primers pairs used were as follows: Ha-Ras forward CAAGAGTGCGCTGACCATCC, reverse CCGGAT CTCACGCACCAAC; Ki-Ras forward AGTGCCTTGACGATACAG, reverse GCATCATCAACACCCTGTCTT; and N-Ras forward GAAA AGCGCACTGACAATCC, reverse CACCACACATGGCAATCCC. For mouse, the primers were as follows: Ha-ras forward CATCCAG CTGATCCAGAACC, reverse CATCTGAATCTTTCACCCGC; Ki-ras forward TACATGAGAACTGGGGAGGG, reverse CTGTCTTGTCTT CGCTGAGG; and N-ras forward CCTGACGATCCAGCTAATCC, reverse ACTTGTTGCCTACCAGCACC. The reverse transcription and amplification reactions were carried out using Promega's Access RT-PCR kit following the manufacturer's instructions using 0.5 μg of total RNA. The annealing temperature used was 59°C. The linear phase of the amplification reaction was identified for each primer pair by varying the cycle number from 10 to 40. Ten percent of the reaction mixture was visualized on an agarose gel stained with ethidium bromide and analyzed with densitometry. For each pair, a cycle number in the linear phase of amplification was used in the experiments to determine the effects of the AS-oligos on mRNA levels for that isoform. As an internal standard, 18S ribosomal RNA primers were used. These were supplied in the Ambion Quantum RNA 18S internal standards kit (Ambion, Whitney, Oxfordshire, UK).

For Western blotting experiments, cells were grown to 50–80% confluence in 35 or 100 mm plates in Dulbecco's modified Eagle's medium/Hams F12 Nut-mix containing 10% fetal calf serum.The cell lysis detergent medium (designated PBSTDS) had the following composition: 1 × PBS, 1% Triton-X 100, 0.5% deoxycholate, 0.1% SDS, leupeptin 0.5 μg/ml, pepstatin 1.0 μg/ml, ethylenediaminetetraacetic acid 1.0 mM and phenylmethylsulfonyl fluoride 0.2 mM. Cells were either lysed at this point or, for experiments involving the

AS-oligos, they were transfected for 16 h with 200 nM oligo in OptiMEM. Following transfection, the medium was removed and replaced with Dulbecco's modified Eagle's medium/Hams F12 Nutmix containing 10–20% fetal calf serum. After 24 h, the cells were washed in PBS and lysed. Nuclear debris was removed by centrifugation at 2300 *g* for 15 min at 4°C.

Cell lysates were assayed for protein content by the Pierce protein assay, and equal amounts of protein were made up to a uniform volume of Western sample buffer comprising the following: (6 × concentrate) 0.5 M Tris (pH 6.8), 0.35 M sodium dodecyl sulfate, glycerol 30%, 0.6 M dithiothreitol and 0.175 M bromphenol blue. Five percent 2-mercaptoethanol was added immediately before loading, and samples were heated to 100°C for 3 min; then, 10 μg protein was loaded onto a 4/15% discontinuous polyacrylamide gel. After transfer, protein detection was performed using monoclonal antiobodies as follows: p21 (1:200 dilution; PharMingen, San Diego, CA), Akt-P-S (Ser473) (1:1000 dilution; Cell Signaling Technology, Beverly, MA) and anti-p27Kip1 antibody (1:1000 dilution; Transduction Laboratories, Lexington, MA). Details of these methods have been reported previously.[21,22]

After incubation with an appropriate secondary antibody coupled to horseradish peroxidase, blots were washed again for 25 min and the detection was carried out using the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Amersham, UK).

Statistics

Statistical analysis was performed using analysis of variance followed by Dunnetts test of significance or an unpaired *t*-test using Prism software (Graphpad, San Diego, CA).

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